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Immunomodulation of Human Peripheral Blood Mononuclear Cell Functions by Defined Lipid Fractions of *Mycobacterium avium*

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Mycobacterial fractions, some of which are associated with the cell envelope of *Mycobacterium avium* serovar 4, were assessed for their ability to affect various immunological functions of human peripheral blood mononuclear cells (PBM). Treatment of PBM with a total lipid fraction derived from *M. avium* serovar 4 resulted in a significant suppression of lymphoproliferative responsiveness to phytohemagglutinin stimulation at concentrations not affecting cell viability. Although a similar suppression was not observed when PBM were treated with purified serovar 4-specific glycopeptidolipids (GPL), treatment with the β-lipid fragment derived from the GPL did result in a significant suppression of phytohemagglutinin responsiveness. Further studies revealed that the total lipid fraction and the β-lipid fragment were effective at significantly reducing the ability of human macrophages to restrict the intracellular growth of mycobacteria and at stimulating PBM to secrete prostaglandin E_2. These same effects were not observed when purified GPL or the reduced oligosaccharide fragment of the GPL was used. Other studies revealed that the total lipid and purified GPL fractions were effective at stimulating tumor necrosis factor alpha release from human PBM, whereas the β-lipid fragment was not. These results indicate that mycobacterial lipids have various immunomodulatory capabilities, depending upon their chemical nature and ability to interact with certain host cells.

The *Mycobacterium avium* complex has not historically been a major clinical concern; however, with the recent appearance of the human immunodeficiency virus, that attitude has shifted to a realization that infections by these mycobacteria can pose a serious medical problem, particularly in individuals whose immune responsiveness has been severely impaired. Although the specific mechanisms that define pathogenicity for the *M. avium* complex have not been clarified, it is becoming more apparent that lipids play an important role as immunomodulators in infections caused by these facultative intracellular parasites.

In two earlier studies, we reported that the surface-associated glycopeptidolipid antigens (GPL) of the *M. avium* complex had the ability to suppress the immune responsiveness of murine splenic cells following intraperitoneal injection (5, 15). Although the GPL were able to affect immune responsiveness in vitro, they did not appear to do so as well as when they were injected intraperitoneally (5). In an effort to explain the results, we hypothesized that the GPL had been degraded and that their metabolites were responsible for the augmented immunosuppression observed in vivo (5). Subsequently, Tsuyuguchi et al. (29) reported that *M. avium* lipids are capable of modulating immune responsiveness by interfering with the ability of human monocytes to express certain accessory molecules that might be involved in nonspecific interactions between monocytes and T cells.

As a continuation of our studies on the immunomodulatory properties of lipids, we recently reported that the β-lipid fragment, which is chemically derived from the GPL, is the active moiety with regard to its capacity to suppress lymphoproliferative responses (27). On the other hand, the oligosaccharide moiety appears to have no effect on the lymphoproliferative responses of murine splenic cells (27), and even though native GPL can be immunosuppressive in vitro (5), they are not as effective as the β-lipid fragment in suppressing mitogen-induced lymphoproliferation (21). This fact has led us to suspect that lipids derived from *M. avium* may differ in their capabilities to affect immunologically important cells and suggests that the chemical nature of a lipid is important as a determining factor in that lipid's ability to interact with and alter the function of those cells.

To further examine the role of *M. avium* lipids in pathogenicity, we report here on the immunomodulatory capabilities of defined lipid fractions derived from serovar 4 of the *M. avium* complex and the reduced oligosaccharide (r-olig) fragment derived from purified GPL. A total lipid fraction, a purified GPL fraction, and a β-lipid fraction were compared along with the r-olig fragment with regard to their ability to modify the lymphoproliferative response of human peripheral blood mononuclear cells to phytohemagglutinin (PHA) and their ability to affect the phagocytic capacity of human peripheral blood macrophages. In addition, the lipid fractions were compared with regard to their ability to stimulate the secretion of two important immunomodulatory substances, prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNF-α).

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MATERIALS AND METHODS

Mycobacteria. *M. avium* complex serovar 4 (TMC 1463) was provided by the National Jewish Hospital and Research Center (Denver, Colo.) through Darrel Gwinn, National Institute of Allergy and Infectious Diseases (Bethesda, Md.). Mycobacteria were cultivated by use of 7H9 Middlebrook broth or agar (Difco Laboratories, Detroit, Mich.) which had been supplemented with glycerol and oleic acid-albumin-glucose (Difco) as described previously (31).

Extraction and purification of lipids and oligosaccharide. Total lipids were extracted from lyophilized mycobacteria with chloroform-methanol (2:1) and processed by means of the Folch procedure as previously described (27, 31, 32). Individual lipid fractions were purified by means of a short column chromatographic procedure as previously described (9). Lipid fractions were examined by development in chloroform-methanol-water (60:12:1; solvent A) or chloroform-methanol (11:1; solvent B), and GPL were identified by reaction with orcinol-sulfuric acid (9). Preparation of the β-lipid and r-olig was achieved by means of the alkaline-catalyzed β-elimination procedure originally described by Brennan and Goren (3) and subsequently used by us to obtain the β-lipid and r-olig from the GPL of *M. avium* serovar 4 (27). Mycobacterial fractions (at concentrations equivalent to those used in this study) were tested for endotoxin activity by means of the *Limulus* amebocyte lysate kit (Sigma) and found to contain ≤0.03 endotoxin unit (≤0.005 ng) of endotoxin per ml.

In some experiments, radiolabeled lipid fractions were purified by the same procedure to monitor the contents of the lipid samples. In such cases, radiolabeled lipid fractions were applied, in parallel, to thin-layer chromatographic (TLC) plates at concentrations ranging from 100 to 300 μg and then developed in either solvent A or solvent B. Following development in the appropriate solvent, centimeter sections were scraped into vials and radioactivity was measured in EcoLite scintillation fluid (WestChem). Adjacent lanes were sprayed with an orcinol-sulfuric acid reagent to confirm the presence or absence of GPL. In addition, radiolabeled lipids were also assayed by means of high-pressure liquid chromatography (HPLC) to confirm the presence of absence of GPL in various fractions. In such cases, lipids were separated on a Beckman Ultrasphere 5-μm spherical 80-Å-pore-size SI analytical column with Beckman System Gold as described previously (27, 32). Samples were injected at a concentration of 800 μg and separated in a mobile phase of 100% chloroform for 10 min and then in a 40-min gradient of 0 to 10% methanol in chloroform at a flow rate of 1.0 ml/min. Following the use of the gradient, 10% methanol in chloroform was maintained for 10 min, and then a gradual return to 100% chloroform took place over 10 min (27, 32).

HPLC data analysis. As stated above, Beckman System Gold was used for computer analysis, after which graphs were produced by a procedure described by Burrier (6). Sample files for each HPLC analysis were analyzed and subsequently translated to .DIF files by use of the File Management utility in System Gold (6). Once the files had been translated to .DIF files, they were loaded into a Macintosh PowerBook 140 (Apple Computer, Cupertino, Calif.) and then converted into graphic form by use of the Excel (Microsoft) program. Graphs were then output by use of a Laserwriter Plus (Apple Computer). To facilitate printing, portions of HPLC runs were deleted on the graph. These portions of the analysis did not contain any radiolabeled lipid components.

Human PBM. Human peripheral blood mononuclear cells (PBM) were derived by use of the buffy coat fraction of whole blood from normal donors as described previously (23). The mononuclear layer (buffy coat) was obtained from normal donors, and viability was determined by means of the trypan blue dye exclusion test to be ≥97%. For some TNF-α induction experiments, platelets were removed by centrifugation over fetal calf serum (FCS) in a manner similar to that described by Merino et al. (19). Aliquots of the PBM suspension at 2×10^7/ml in RPMI 1640 were layered over threefold volumes of FCS and centrifuged at $125 \times g$ for 15 min. The cell pellet was then resuspended in RPMI 1640 with 10% heat-inactivated FCS (RPMI complete), and the cell count was adjusted to 10^6 viable cells per ml by trypan blue exclusion.

Application of lipids. Two methods were used to expose human cells to lipid fractions. For human cells which were examined for phagocytic activity and cells which were examined for PGE_2 and TNF-α, lipids were first dissolved in HPLC-grade chloroform (Fisher) and appropriate concentrations were then applied to previously sterilized glass coverslips which had been placed in culture wells. Lipid solutions were allowed to dry prior to the addition of human cells. Appropriate controls included coverslips to which equivalent amounts of solvent had been applied. For lymphoproliferative assays, lipid fractions were first dissolved in ethanol-hexane (5:4) and then applied to the bottom of wells in 96-well microdilution plates. The plates were then dried under a biological safety hood prior to the addition of human cells. As above, appropriate controls included wells to which equivalent amounts of solvent had been applied.

Intracellular growth experiments. Human macrophage monolayers were infected with *M. avium* serovar 2 as described previously (23). The macrophages were allowed to phagocytize the bacteria for 4 h at 37°C, after which all the extracellular bacilli were thoroughly washed away with Hanks balanced salt solution. The number of bacteria effectively phagocytized was determined by lysing the macrophages with 0.25% (wt/vol) sodium dodecyl sulfate, doing immediate serial dilutions, and plating the lysates on 7H11 agar medium for viable count determinations. The addition of 0.25% sodium dodecyl sulfate does not lower bacterial viable counts (23). After phagocytosis, the number of viable bacteria was also enumerated by lysing the macrophages at various time intervals, up to 5 days, and plating the bacteria on 7H11 agar medium. The results were compared with the growth of bacteria in the control culture (untreated macrophages).

Viability assay. Viability assays were conducted as described previously, with a few modifications (27). Lipid fractions were first dissolved in ethanol-hexane (5:4) and then applied to the bottom of microdilution plates as described above. r-olig was dissolved in sterile distilled pyrogen-free water prior to addition to wells (27). In all cases, additional wells were treated with equivalent amounts of solvent to obtain appropriate control values for data compilation. Human PBM were added to individual wells to achieve concentrations ranging from 5 to 100 μg of the fraction being tested per 4×10^5 cells. Following 2, 24, and 48 h of exposure, the viability of cells was determined by trypan blue dye exclusion as described previously (27).

Lymphoproliferative assay. Human PBM (see above) were adjusted to 4×10^6 viable cells per ml in RPMI complete and distributed to 96-well microdilution plates (Costar) at a final

concentration of 4×10^5 cells per 100 μl per well. Culture plates were incubated for 2 h at 37°C in 5% CO_2, and then PHA (Wellcome Research Laboratories, Beckenham, England), diluted in RPMI complete, was added to wells to achieve a final volume of 200 μl. Preliminary experiments were performed to determine the minimum concentration of PHA that produced the optimum response; this concentration was then used for these experiments. Control wells received RPMI complete only. Cultures were incubated for 66 h and then pulsed with 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; ICN Pharmaceuticals, Irvine, Calif.) for the last 6 h. Cells were harvested and counted in Ecolume scintillation fluid (ICN) (27). Samples were assayed in triplicate, and suppression was determined as described previously (5, 15, 27). For statistical analysis, the mean for each group of cells was compared with the mean for the untreated (control) group of cells. Control wells, previously treated with equivalent amounts of solvent, did not produce significantly different results.

PGE_2 assay. PGE_2 was measured by means of a radioimmunoassay (Pasteur Diagnostics, Paris, France) as described previously (25). Supernatants derived from culture wells and lysed macrophages were passed through a 0.22-μm-pore-size filter and then diluted in 10 mM phosphate-buffered saline (PBS) (pH 7.4) along with antibody and ^{125}I-radiolabeled antigen. Bound fractions were precipitated with polyethylene glycol 6000 (30% [wt/vol]) and then centrifuged (2,000 × *g*, 10 min, 4°C). The resulting pellets were assayed for radioactivity, and the results were expressed as picograms of total PGE_2 synthesized per 10^6 cells.

TNF-α bioassay. For experiments involving TNF-α induction, lipids were applied to coverslips as described above, with the following exceptions. Coverslips of 15-mm diameter were sterilized with dry heat (180°C, 4 h) and then placed in individual wells of a 24-well culture plate (Costar). Lipids were applied in chloroform and allowed to dry prior to the addition of 2 ml of cell suspension (2×10^6 PBM per well). Following incubation for 24 h at 37°C in 5% CO_2, culture supernatants were collected and stored at −20°C prior to assay. The bioassay for TNF-α was based on the mouse target cell line L929, and the procedure was developed according to previous studies by Valone et al. (30) and Flick and Gifford (11). The L929 cells (a kind gift from Andrew Paquet, Jr., Texas Christian University, Fort Worth) were cultured in RPMI complete and subcultured at 3-day intervals. Confluent cells in flasks were trypsinized (trypsin-EDTA; GIBCO), washed twice (200 × *g*, 5 min), counted with a model ZM Coulter Counter, and adjusted to 3×10^5 cells per ml in RPMI complete. The cells were then plated at 100 μl per well in 96-well plates and incubated overnight (37°C, 5% CO_2). Culture supernatants from PBM were thawed and centrifuged briefly in a microcentrifuge (15,000 × *g*, 2 min) prior to dilution in RPMI 1640–0.5% FCS containing 2 μg of actinomycin D per ml. Diluted samples along with appropriate dilutions of a recombinant human TNF-α standard (R&D Systems) were then added at 100 μl per well, and the plates were incubated for 20 h prior to the addition of 50 μl of 0.033% neutral red in PBS (GIBCO 630-5330; 1:10 in PBS). After incubation for 1 h, the wells were aspirated and washed twice with 250 μl of warm PBS. The dye was extracted for 10 min with ethanol–0.1 M monobasic sodium phosphate (1:1). The A_{540} was measured by use of a Dynatech MR 600 enzyme-linked immunosorbent assay (ELISA) reader, and a standard curve was constructed. The sample values were then obtained from linear regression analysis of the semilog plot.

ELISA for TNF-α. A two-site sandwich ELISA for TNF-α was developed by use of a monoclonal capture antibody (Upstate Biotechnology, Inc.) and a polyclonal immunoglobulin G fraction of rabbit antisera (Sigma). The monoclonal antibody (immunoglobulin G1) at 500 ng per well, was used to coat Immulon 1 Removawells (Dynatech) overnight at room temperature. The wells were then postcoated with 0.01% casein in PBS (30a). Culture supernatants or a recombinant human TNF-α standard was diluted in 0.05% bovine serum albumin–PBS and incubated in the wells for 1 h at 37°C; this step was followed by three washes in 0.005% casein–PBS. Rabbit antibody was then added at 2.5 μg/ml and incubated for 1 h at 37°C, and the plates were washed again prior to the addition of a peroxidase conjugate of goat anti-rabbit immunoglobulin G (Fab_2) (Zymed). Following another incubation period, the plates were washed with casein-PBS before the addition of 3,3′,5,5′-tetramethylbenzidine in 0.1 M sodium acetate–citric acid containing 0.005% hydrogen peroxide. Color development proceeded for 30 min at room temperature prior to termination with 50 μl of 2 M sulfuric acid per well. The A_{450} was measured by use of the Dynatech ELISA reader with a reference wavelength of 570 nm. A standard curve was constructed, and the sample concentrations were derived from linear regression analysis of a log-log plot.

Statistical analysis. The significance of differences between groups was determined by a one-way analysis of variance (ANOVA), and correction for multiple comparisons (post-test) was done by means of the Bonferroni method with InStat software for MacIntosh (GraphPad Software, San Diego, Calif.).

RESULTS

Mycobacterial fractions. Four mycobacterial fractions were used in this investigation: total lipids, purified GPL, β-lipid fragment from GPL, and the r-olig fragment from GPL. The total lipid fraction contains the total lipids extractable by means of the Folch procedure. This fraction would essentially include noncovalently linked lipids uncontaminated with nonlipid components, such as free sugars and amino acids (16). Purified GPL were obtained by further elution with 4 to 7% methanol in chloroform (9). Structural analysis of the GPL has been reported elsewhere (3, 13). The GPL can be identified by their characteristic yellow-gold color reaction to orcinol following TLC separation (Fig. 1, lanes A and B). The GPL can also be identified by the HPLC pattern obtained by analyzing [^{14}C]Phe-radiolabeled lipids (total lipid fraction in Fig. 2). In addition, the total lipid fraction contains the apolar GPL, which have a structure similar to that of the GPL, with the exception that the oligosaccharide is replaced with 6-deoxytalose (3, 4, 13). The apolar GPL have a retention time of between 30 and 35 min, similar to that of the β-lipid fraction. Also present in the total lipid fraction is a group of phenylalanine-containing lipopeptides, some of which are partially glycosylated and others of which are nonglycosylated (2). An example of one of these lipopeptides would be the first major peak presented in the HPLC pattern of the total lipid fraction (Fig. 2, retention time of 27 min). This lipopeptide has been reported by us for both rough- and smooth-colony variants of serovar 4 and contains phenylalanine, alanine, and isoleucine but no carbohydrate (2). A detailed analysis of these lipids is currently in progress.

Preparation and detailed chemical analysis of the β-lipid and r-olig fractions have been described elsewhere and will

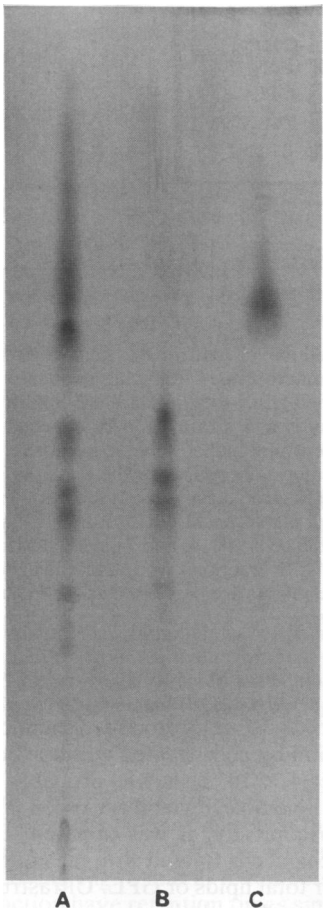

FIG. 1. Thin-layer chromatogram representing the total lipid (A), purified GPL (B), and β-lipid (C) fractions obtained from *M. avium* serovar 4. Samples were developed in solvent A and sprayed with orcinol-sulfuric acid to detect GPL.

therefore not be repeated here (27). In brief, the β-lipid fragment is derived by alkaline-catalyzed β-elimination of the oligosaccharide component from GPL (3) and is primarily a lipopeptide fragment containing a terminal sugar: fatty acyl-D-Phe-α-aminobutyric acid-D-Ala-L-alaninol-3,4-di-*O*-methyl-rhamnose. The resulting r-olig fragment from serovar 4 is a tetrasaccharide with the following structure: 6-deoxy-talitol-rhamnose-2-*O*-methyl-fucose-4-*O*-methyl-rhamnose (18). Previously, using murine splenic cells, we demonstrated that the β-lipid fragment and not the r-olig fragment is immunosuppressive with regard to mitogen-induced lymphoproliferative responses (27).

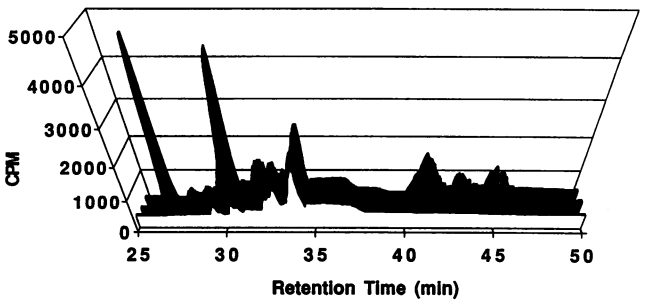

FIG. 2. HPLC separation of [^{14}C]Phe-radiolabeled total lipid fraction (■), purified GPL fraction (▩), and β-lipid fraction (□) from *M. avium* serovar 4. Column conditions were as described in text.

To further define these lipid fractions, parallel batches of [^{14}C]Phe-radiolabeled lipids were processed and analyzed by means of TLC and HPLC procedures. TLC analysis of the radiolabeled lipids from individual fractions revealed a complete absence of the GPL in the purified β-lipid fraction. This result was also confirmed when we sprayed plates with the orcinol-sulfuric acid reagent and did not observe the characteristic yellow-gold spots associated with the GPL. Analysis of radiolabeled lipids by HPLC further confirmed the absence of the GPL in that fraction and helped to define the composition of the fractions with regard to [^{14}C]Phe incorporation (Fig. 1).

Effects of lipids on the viability of human mononuclear cells. Cells were exposed to individual lipid fractions or r-olig for 2, 24, and 48 h, after which cell viability was determined by trypan blue exclusion. For the total lipid and purified GPL fractions, viability did not fall below 90%, even after up to 48 h of treatment with up to 50 μg of lipid per 4×10^5 cells. Viability was not affected by the β-lipid fraction after 2 or 24 h of exposure for all concentrations or after 48 h for the 10-μg concentration. Viability was slightly affected by treatment with the β-lipid fraction after 48 h in that treatment with 20 and 50 μg per 4×10^5 cells reduced viability to 79% (standard deviation, ±5%) and 83% (standard deviation, ±7%), respectively. The viability of nontreated cells was 93% (standard deviation, ±3%) at 48 h.

Lymphoproliferative responses of cells exposed to mycobacterial components. With respect to the r-olig fraction, no suppression of the lymphoproliferative response to PHA was observed following 2 h of treatment with 10, 20, or 50 μg of r-olig per 4×10^5 cells (data not shown). Likewise, treatment with the purified GPL fraction at equivalent concentrations had no significant effect on the lymphoproliferative response to PHA (Fig. 3B). However, treatment with the total lipid and β-lipid fractions resulted in a significant immunosuppression of the lymphoproliferative response to PHA stimulation (Fig. 3A and C, respectively), with suppression ranging from 26 to 91% for the total lipid fraction (10 to 50 μg) and 57 to 99% for the β-lipid fraction (20 to 50 μg).

Effects of mycobacterial fractions on the intracellular growth of *M. avium*. Human PBM were added to wells that contained coverslips to which lipids had previously been applied. One hundred micrograms of lipid was applied to each coverslip, and 2×10^6 cells were added to each well to achieve a final concentration of 100 μg/2×10^6 cells. This concentration is equivalent to 20 μg/4×10^5 cells, the minimal concentration that had resulted in significant suppression of PHA-stimulated lymphoproliferative responses for the total lipid and β-lipid fractions (see above). Exposure of human macrophages to the total lipid fraction resulted in a marked decrease in the ability of the cells to restrict the growth of mycobacteria (Fig. 4). With respect to the various fractions, treatment with the β-lipid fragment resulted in a significant decrease in the ability of the macrophages to restrict the growth of mycobacteria, whereas treatment with purified GPL or the r-olig fragment did not (Fig. 4). Thus, the only fractions that interfered with the ability of the macrophages to restrict the growth of mycobacteria were the total lipid and β-lipid fractions. To obtain a possible explanation for the ability of these two fractions to suppress PHA responsiveness and affect macrophage function, we next examined the potential of the various mycobacterial fractions to stimulate PGE_2 secretion.

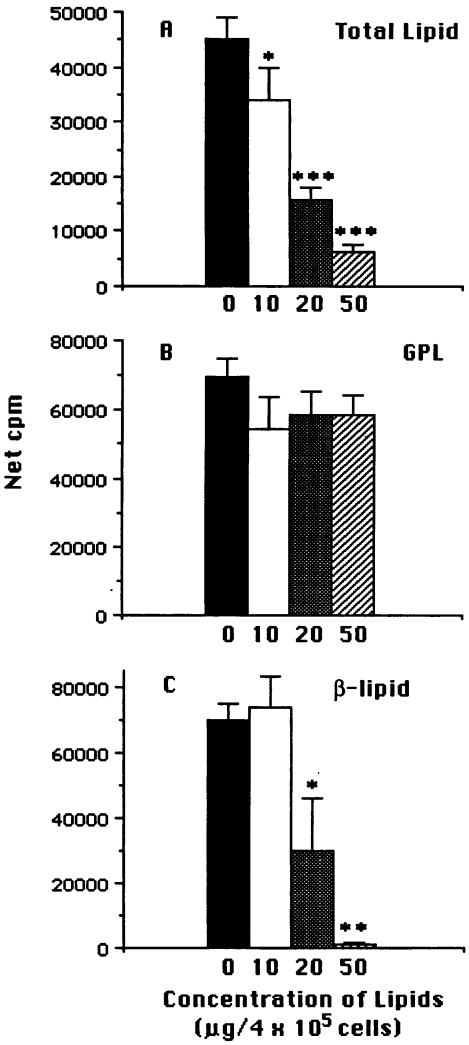

FIG. 3. Lymphoproliferative responses of human peripheral blood mononuclear cells to PHA stimulation. Cells were treated with the total lipid (A), purified GPL (B), or β-lipid (C) fraction obtained from *M. avium* serovar 4 at a concentration of 0 (■), 10 (□), 20 (▩), or 50 (▨) μg per 4 × 10^5 cells. The mean value for each group was compared with that of its control (i.e., 0 μg, ■). Significant differences among the means were determined by an ANOVA with the Bonferroni method to correct for multiple comparisons. *P* values were ≤0.05 (*), ≤0.01 (**), and ≤0.001 (***).

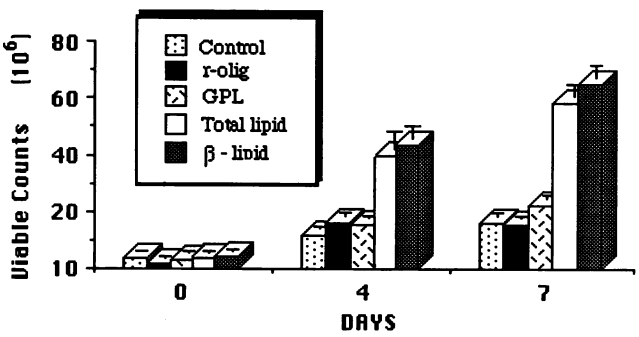

FIG. 4. Intracellular growth of *M. avium* serovar 2 in human peripheral blood macrophages following exposure of macrophages to the r-olig, purified GPL, total lipid, and β-lipid fractions obtained from *M. avium* serovar 4. Controls were exposed to coverslips to which an equivalent amount of solvent had been applied (solvent controls). Nontreated controls were the same as solvent controls (data not shown). Macrophages were lysed with Triton X-100, and mycobacteria were plated on Middlebrook 7H10 agar to determine the number of viable cells at 0, 4, and 7 days following infection. The final concentrations of fractions were equivalent to 20 μg/4 × 10^5 macrophages. The results are presented as the mean ± the standard error for three assays.

PGE_2 release from cells exposed to mycobacterial components. Human PBM were exposed to the same concentrations of lipids and r-olig as those used for the phagocytic assay, i.e., 100 μg of lipid per 2 × 10^6 cells (equivalent to 20 μg/4 × 10^5 cells). Exposure of human PBM to the total lipid or β-lipid fraction stimulated the cells to secrete PGE_2 in significant amounts, compared with those in control samples (Fig. 5). A similar treatment with equivalent amounts of purified GPL or r-olig, however, did not result in PGE_2 secretion (Fig. 5).

TNF-α release after exposure to mycobacterial components. Treatment with either total lipids or GPL at concentrations of 50, 100, and 250 μg/2 × 10^6 cells (equivalent to 10, 20, and 50 μg/4 × 10^5 cells, respectively) resulted in significant amounts of TNF-α secretion from human PBM (Fig. 6). Although TNF-α was produced by PBM treated with the β-lipid fraction, only cells treated with the equivalent of 50 μg of β-lipid per 4 × 10^5 cells produced significant quantities. TNF-α production by cells treated with the equivalent of 50 μg of β-lipid per 4 × 10^5 cells was probably augmented by the effect of the lipid on cell viability. When those PBM were observed microscopically, it was apparent that they were more damaged than cells treated with the equivalent concentrations of either total lipids or GPL. Ultrastructural damage of murine splenic cells with this concentration of β-lipid has been reported by us elsewhere (21, 27). Comparatively, both the total lipid and the GPL fractions were far more effective at inducing TNF-α than the β-lipid fraction. Exposure of PBM to the same concentrations of r-olig did not result in TNF-α secretion (data not shown). In addition, parallel studies with the L929 cell bioassay demonstrated that the TNF-α secreted in response to treatment with lipid fractions was biologically active (data not shown).

DISCUSSION

When human PBM were exposed to mycobacterial fractions at concentrations not affecting viability, it was observed that although the total lipid and β-lipid fractions affected the lymphoproliferative responses in a dose-dependent manner, neither the purified GPL fraction nor the r-olig fraction did so. Previously, using mouse splenic cells, we reported that the β-lipid fragment is the immunosuppressive fragment of the GPL, whereas the r-olig fragment is not (27). In the present study, we further confirm those results by using human cells and, in addition, observe that the total lipid and β-lipid fractions affect the ability of human macrophages to control the growth of mycobacteria, whereas the purified GPL and r-olig fractions do not. The total lipid and β-lipid fractions are also responsible for causing the release of high levels of PGE_2, which is probably one explanation for their ability to affect the function of macrophages.

Although the β-lipid fragment is a chemically derived fraction, it does have a structure similar to those of previously reported lipopeptides, which are more apolar than the GPL and have HPLC retention times closer to that of the

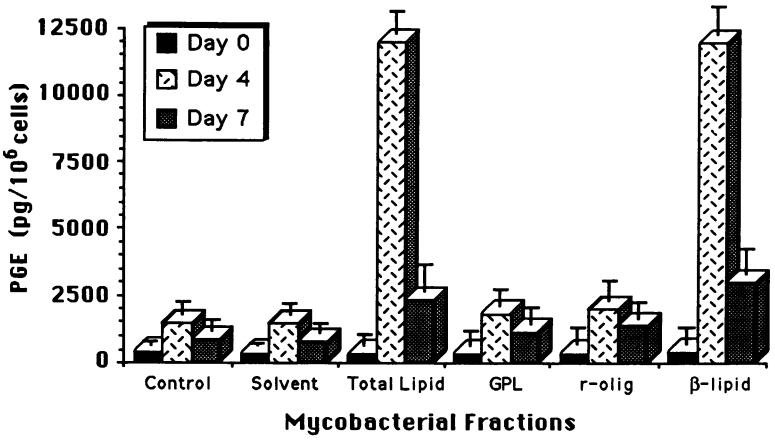

FIG. 5. Secretion of PGE_2 from human peripheral blood mononuclear cells exposed to the equivalent of 20 μg of total lipid, purified GPL, β-lipid, or r-olig fraction from *M. avium* serovar 4 per 4×10^5 cells. Controls consisted of nontreated cells (control) and nontreated cells exposed to coverslips previously treated with chloroform (solvent). Cells were exposed to mycobacterial fractions for 0, 4, and 7 days prior to the PGE_2 assay of supernatants. Results are reported as the mean ± the standard deviation for two experiments.

β-lipid fragment (Fig. 2) (2). Because of this structural similarity, the β-lipid fragment was used in this investigation to represent similar "apolar" lipids present in the total lipid fraction. By studying Fig. 1 and 2, it can be seen that lipids in the β-lipid fraction have retention times similar to those of other phenylalanine-containing lipids in the total lipid fraction. In fact, the most obvious difference between the β-lipid fraction and the total lipid fraction is the complete lack of GPL, which do not affect lymphoproliferative responses or phagocytic ability or cause the release of PGE_2. These findings suggest that lipids which are more apolar in nature may be more likely to affect the stimulation of PGE_2, possibly because of a greater ability to perturb membrane systems. This proposed mechanism of lipid-membrane interaction has previously been suggested with regard to mycobacterial lipids such as the C-mycosides (i.e., apolar GPL) (17, 26) and phenolic glycolipids (12).

Our findings may also help to explain the varied reports regarding the immunosuppressive ability of *M. avium*. Edwards et al. (10) reported that the release of PGE_2 associated with chronic infection involving *M. intracellulare* could be reversed with indomethacin, muramyl dipeptide, or gamma interferon (IFN-γ). Shiratsuchi et al. (24) reported that IFN-γ was effective in decreasing mycobacterial growth within human monocytes and that a combination of IFN-γ and indomethacin resulted in a 10-fold decrease in mycobacterial intracellular growth. Although there was no other cytokine or combination of cytokine with indomethacin that achieved similar results, it was reported that other cytokines, such as interleukin-1, -3, and -6 and macrophage colony-stimulating factor, actually increased the growth of certain *M. avium* strains (24). Another study also reported that IFN-γ and indomethacin rendered murine peritoneal macrophages more bacteriostatic than macrophages treated with IFN-γ alone (8). Tsuyuguchi et al. (29) have reported that PGE_2 is not responsible for immunosuppression resulting from the treatment of human cells with either *M. avium* or lipids derived from *M. avium*. A prostaglandin-independent mechanism was suggested by Tomioka et al. (28), but because indomethacin did not completely overcome the suppressive action of the macrophages in their study, those authors concluded that prostaglandins may also play a role in mediating infection by *M. avium*. More recently, we reported that the intracellular growth of *M. avium* in human macrophages is due to the increased synthesis of PGE_2 (22); however, it appears that PGE_2 is not the only immunosuppressive pathway operating in the infectious process, because indomethacin only partially reversed the impairment of normal microbicidal functions in that study. We also recently reported that indomethacin is not effective at removing the immunosuppression resulting from the treatment of mouse splenic lymphocytes with GPL and the β-lipid fragment, but an assay for PGE_2 was not performed in that study (21).

These results, along with those reported in this paper, suggest that immunosuppressive factors in addition to PGE_2 exist and that PGE_2 release varies depending upon the lipids present in the infecting dose. Thus, for example, a strain of *M. avium* producing more apolar lipids might be expected to cause the secretion of more PGE_2, whereas a strain producing a higher concentration of GPL might be expected to cause the secretion of either no PGE_2 or less PGE_2. More specific studies must be performed before a complete assessment can be obtained.

In parallel with the PGE_2 analysis, it is interesting to note that the intracellular growth of *M. avium* was significantly enhanced only in the case of macrophages treated with the total lipid and β-lipid fractions. Indeed, inhibition of the microbicidal mechanisms of bovine PBM-derived macrophages by distinct glycolipid fractions from *M. avium* was recently reported (14). However, the glycolipids used in that study were chemically distinct from our preparations (14). Thus, our results not only show the dual role that may be played by *M. avium* serovar 4 lipid fractions but also demonstrate the importance of mycobacterial lipids in the host responsiveness to an important opportunistic pathogen associated with human immunodeficiency virus-infected individuals.

Considering TNF-α release, it is interesting to note that the purified GPL and total lipid fractions were far more effective at stimulating human PBM to release TNF-α than were the β-lipid and r-olig fractions. This result is similar to what we previously reported for mouse splenic cells, in which both the β-lipid and the GPL fractions were effective at stimulating TNF-α release, but the GPL fraction was about twice as effective as the β-lipid fraction at equivalent

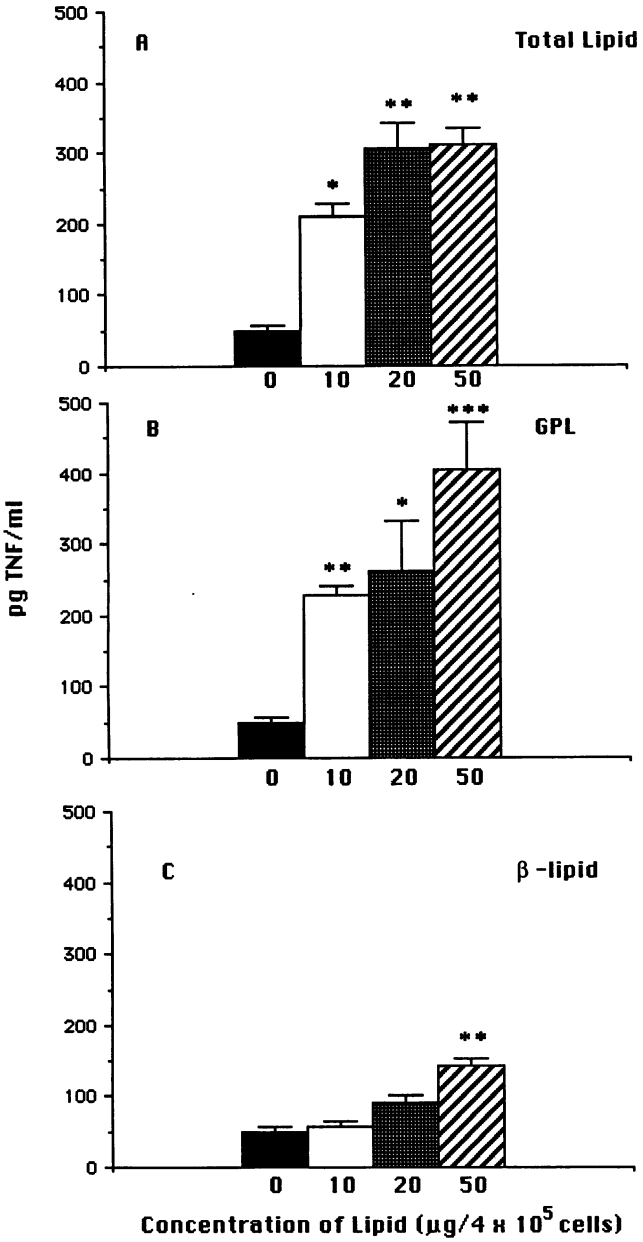

FIG. 6. TNF-α secreted from human peripheral blood mononuclear cells following 24 h of exposure to the equivalent of 0 (■), 10 (□), 20 (■), or 50 (▨) µg of total lipids (A), purified GPL (B), or β-lipid (C) per 4 × 10^5 cells. The quantity of TNF-α is given as picograms per milliliter of culture fluid, as determined by the immunoassay described in the text. Assays were performed in quadruplicate, and results are reported as the mean ± the standard deviation. Results are representative of assays done for three healthy donors. The mean value for each group was compared with that of its control (i.e., 0 µg, ■). Significant differences among the means were determined by an ANOVA with the Bonferroni method to correct for multiple comparisons. *P* values were ≤0.05 (*), ≤0.01 (**), and ≤0.001 (***).

concentrations (20; this paper). Thus, although the total lipid fraction, which contains the GPL, was capable of stimulating TNF-α release in the present study, it was not as effective as the purified GPL fraction. Furthermore, removal of the oligosaccharide moiety from the GPL (i.e., the β-lipid fragment), considerably reduced its ability to stimulate TNF-α secretion. Variable abilities of structurally related mycobacterial lipids to induce TNF-α have also been reported for lipoarabinomannan and lipomannan (7). Apparently, lipoarabinomannan isolated from the avirulent H37Ra strain of *M. tuberculosis* is more potent in its ability to induce TNF-α secretion than lipoarabinomannan isolated from the virulent Erdman strain (7). This variance is probably due to the presence of short mannan segments that mask the arabinan side chains, because lipomannan, which is devoid of arabinose, is only able to induce low levels of TNF-α (7).

As discussed previously, it appears that an explanation for *M. avium* pathogenicity depends, at least in part, upon an understanding of various lipids and their role in immunomodulatory events that take place in the host (1). This current report, therefore, is an important first step in understanding the complex nature of *M. avium* lipids and their role in pathogenicity. In future studies, we hope to further classify and characterize individual *M. avium* lipids with regard to their ability to participate in the apparently complex events that take place in *M. avium* infections and begin to develop a hypothesis regarding those events and their contribution to *M. avium* virulence and pathogenicity.

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